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An adenovirus gene delivery system was developed allowing target gene delivery to the mouse mammary gland and cultured cells. This system was used to express a truncation mutant of the tight junction protein occludin in the mouse mammary gland and in cultured epithelial cells. The expression of truncated occludin caused programmed cell death in the mouse mammary gland epithelium and in cultured epithelial cells. Occludin and the apical junction complex proteins ZO-1 and beta-catenin left the tight junction following transgene expression. The beta-batenin binding, actin remodeling protein fascin was up regulated in transgene expression cells. The fluorescent staining of fascin was brilliant and cytoplasmically diffuse. Fascin upregulation was proposed to be an inherent part of the programmed cell death process. Recent work demonstrated an up-regulation of fascin during cell death downstream of topoisomerase inhibition. That fascin is apparently upregulated downstream of such divergent apoptotic stimuli as loss of cell/cell contact and DNA strand breaks suggests that actin plays a fundamental role in apoptosis. The apical junction proteins beta-catenin, AF-6 and P-120 thought to interact molecularly with fascin, were shown to leave the apical junction complex and distribute to the perinuclear region. Monomeric actin was shown to increase during apoptosis.

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Table of Contents

Introduction:

The tight junction is an array of anastomosing proteinaceous fibers lying in the apicolateral membrane regions of adjacent epithelial cells. These fibers lie in parallel along their lengths and are thought to interact via hetero and homophilic interactions among the occludin and claudin proteins composing these fibers. The C termini of occludin, nectins, and the claudins have been shown to interact with membrane associated guanylate kinases (MAGUKs), such as ZO-1, the first tight junction protein to be cloned. Tight junction associated MAGUKs and other proteins form an intercellular plaque that runs continuously along the tight junction fibrils. This plaque connects transmembrane proteins in tight junction fibrils to actin stress fibers, which in turn connect to the perijunctional actin ring. The tight junction complex lies at the apical boundary of the cadherin based adherens junction complex. The tight junction, the adherens junction, and the perijunctional actin ring comprise the apical junctional complex. The cadherin based adherens junction occurs basal to the tight junction and, like the tight junction, runs continuously along the apicolateral border of adjacent epithelial cells. The mammary adherens junction is largely comprised of the transmembrane protein E-cadherin that forms homophilic interactions between adjacent cells. An intercellular plaque runs along the intercellular C-termini of the cadherin molecules. This plaque is comprised of α-catenin (vinculin), β-catenin, p-120 catenin, AF-6, and γ-catenin (plakoglobin). The catenin-based plaque connects the transmembrane cadherins to actin stress fibers that in turn connect to the perijunctional actin ring (see figure 1).

The architecture of the apical junction complex is almost always perturbed in breast cancer. ZO-1, E-cadherin, all of the catenins, and fascin are very frequently irregularly expressed in breast cancer. The perijunctional actin ring is usually poorly defined in breast cancer. Up-regulation of fascin is a feature of transformed cells in general.

A great deal of scientific research has been devoted to intracellular signaling downstream of cadherin binding events and it has been shown that proper cadherin mediated cell/cell contact is necessary for normal differentiated epithelial cell survival. Based upon my preliminary results from this project, I hypothesize that there is also intracellular signaling from the tight junction and that proper occluding/claudin/nectin mediated cell/cell contact is necessary for mammary epithelial cell survival. The cells comprising an aggressive breast tumor are by definition able to survive improper cell/cell binding. Understanding the survival dependency of healthy epithelial cells upon proper cell/cell contact will be of great value in fighting breast cancer.

With a better understanding of the mechanisms that cause healthy cells to die when unable to maintain proper attachment, it may be possible to develop therapies that only target cells already showing irregular cell/cell binding, rather than targeting all cells that are dividing.

This project is currently testing the hypothesis that mammary epithelial cells require normal occludin mediated tight junction cell/cell attachment for survival. The roles played by the actin bundling protein fascin and the adherens junction proteins β-catenin, P-120 catenin, and AF-6 during programmed cell death are being studied. The roles of these proteins are also being studied during cell death initiated by DNA strand breakage and the normal involution of the gland at the end of a lactation cycle in all mammals.

Body:

During the previous grant cycle and adenovirus gene delivery system was developed and used to deliver a truncated occluding transgene to cells in culture and to the mouse mammary gland epithelium. Cells expressing the transgene underwent programmed cell death. The actin remodeling protein fascin appeared to play a significant role in this process.

The grant was then discontinued until March of this year, when I resumed my graduate training. Since that time, I have been studying fascin and several apical junction proteins during cell death using two different cell death models: topoisomerase inhibition in cultured cells and more recently cells undergoing programmed cell death during mouse mammary gland involution following pup removal.

1. It was first necessary to develop a drug cocktail that would cause cultured mammary epithelial cells to undergoe anoikis (epithelial apoptosis) without wiping out the cultures entirely. Figure 1 shows that some cells are undergoing apoptosis while others survive. Cells still maintain high transepithelial electrical resistance for three days following treatment. This is an indicator of epithelial integrity. This should be necessary as mammary epithelial cells undergo apoptosis in coordination with surviving neighbor cells in the body.
Conclusion: I have developed a drug cocktail that induces apoptosis in a sufficient number of cells without destroying the epithelial properties necessary to anoikis.

2. I have been treating filter-grown cells with topoisomerase inhibitors and staining for the following proteins:

Fascin: Figure 2 shows the up-regulation of fascin in cells 72 hours following drug treatment. This staining pattern is quite different from that of fascin downstream of the occludin transgene expression (figure 3).

This leads to the hypothesis that fascin may act differently during cell death downstream of different signaling pathways.
Beta-catenin: Figure 4 shows the distribution of beta-catenin downstream of topoisomerase inhibition. This is again a very different staining pattern than that observed downstream of truncated occludin. The TUNEL staining shows dying or pycnotic nuclei (compare figures 3 and 4). Beta-catenin is widely studied and has a dual role in contact dependent cell survival and in cell death.
AF-6: Figure 5 shows the staining of the raps target, tight junction protein AF-6. AF-6 is thought to modulate fascin activity through the Rac/Rho pathway. AF-6 was recently shown to play a role in preventing proper cadherin function when the AF-6 transmembrane tight junction binding partner nectin is blocked with self peptide.
p-120 catenin: Figure 6 shows the staining of p-120 catenin, also thought to modulate fascin activity through the Rac/Rho pathway. Like beta-catenin, P-120 is thought to have a dual role in cell survival and death.
Conclusion: I have demonstrated that the distribution of proteins during cell death varies with the mechanism of induction

3. I have recently been collecting tissues from mouse mammary glands at various time points following pup removal on the ninth day of lactation. Cells begin shedding into the lumen at 48 hours.Figure 7 shows a cell shed into the mammary lumen stained with an antibody to activated caspase-3. **CONCLUSION:** I can collect the mammary glands from involuting mice at various time points following pup removal to get different stages of truly natural programmed cell death.

List of Key Research Results:

- I have confirmed the feasibility of fascin immunofluorescence in a mammary epithelial cell line pertaining to task 1. My preliminary evidence concerning the role of fascin during apoptosis downstream of tight junction disruption seems to be of greater relevance to medical research than looking at fascin behavior in transgenic mice with lactational defects. Instead of collecting tissues from *patched* mice I am now rather looking at the behaviour of fascin in the wild type system during natural mammary involution and during programmed cell death downstream of tight junction perturbation.
- I have demonstrated my ability to construct adenovirus vectors and use them in the transduction of the mouse mammary gland epithelium pertaining to task 2. I am, however, now planning to use this technology to construct adenoviruses geared towards tight junction disruption as is the case with the truncated occluding virus.
- Because of the finding that tight junction perturbation causes cell death, task 3 is no longer an advisable line of research.
- I have demonstrated dynamic redistribution of five apical junction proteins (ZO-1, fascin, AF-6, P120-catenin, and β-catenin) in response to perturbation of the tight junction pertaining to task 4. I propose to do the work in task 4 with viruses designed to perturb the apical junction complex.

List of Reportable Outcomes:

- Poster presentation at DOD Era of Hope 2002 in Orlando (see enclosed abstract).
  Transduction of the Mammary Epithelium with Adenovirus Vectors In Vivo. T. Russell, A. Fischer, N. Beeman, E. Freed, M. Neville, and J. Schaack. J Virol, 2003: 5801-5809.

Conclusions:

An adenovirus gene delivery system was developed allowing target gene delivery to the mouse mammary gland and cultured cells. This system was used to express a truncation mutant of the tight junction protein occludin in the mouse mammary gland and in cultured epithelial cells. The expression of truncated occludin caused programmed cell death in the mouse mammary gland epithelium and in cultured epithelial cells. Occludin and the apical junction complex proteins ZO-1 and beta-catenin left the tight junction following transgene expression. The beta-catenin binding, actin remodeling protein fascin was up regulated in transgene expressing cells. The fluorescent staining of fascin was brilliant and cytoplasmically diffuse. Fascin upregulation was proposed to be an inherent part of the programmed cell death process. Recent work demonstrated an up-regulation of fascin during cell death downstream of topoisomerase inhibition. That fascin is apparently upregulated downstream of such divergent apoptotic stimuli as loss of cell/cell contact and DNA strand breaks suggests that actin plays a fundamental role in apoptosis. The apical junction proteins beta-catenin, AF-6 and P-120 thought to interact molecularly with fascin, were shown to leave the apical junction complex and distribute to the perinuclear region. Monomeric actin was shown to increase during apoptosis.

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Transduction of the Mammary Epithelium with Adenovirus Vectors In Vivo

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Because the mammary parenchyma is accessible from the exterior of an animal through the mammary duct, adenovirus transduction holds promise for the short-term delivery of genes to the mammary epithelium for both research and therapeutic purposes. To optimize the procedure and evaluate its efficacy, an adenovirus vector (human adenovirus type 5) encoding a green fluorescent protein (GFP) reporter and deleted of E1 and E3 was injected intraductally into the mouse mammary gland. We evaluated induction of inflammation (by intraductal injection of [^{14}C]sucrose and histological examination), efficiency of transduction, and maintenance of normal function in transduced cells. We found that transduction of the total epithelium in the proximal portion of the third mammary gland varied from 7% to 25% at a dose of 2×10^6 PFU of adenovirus injected into day 17 pregnant mice. Transduction was maintained for at least 7 days with minimal inflammatory response; however, significant mastitis was observed 12 days after transduction. Adenovirus transduction could also be used in the virgin animal with little mastitis 3 days after transduction. Transduced mammary epithelial cells maintained normal morphology and function. Our results demonstrate that intraductal injection of adenovirus vectors provides a versatile and noninvasive method of investigating genes of interest in mouse mammary epithelial cells.

The mammary gland is a compound lobulotubular structure that is a reliable model for developmental studies of cellular growth and differentiation, epithelium-stroma interactions, and tissue-level analysis of systemic hormonal regulation (13). In humans, it begins to develop in the 18- to 19-week fetus and after birth remains quiescent until puberty, when hormonal stimulus by estrogen and growth hormone triggers the tree-like branching of a network of ducts that extends from the nipple into the mammary fat pad on the anterior wall of the thorax (12, 13). Lobular structures, which will become the milk-secreting acini, originate from these ducts. Lobular development is highly dependent upon hormonal stimulation, and in humans, in whom these lobular structures are known as terminal duct lobular units, begins after the onset of menses. In mice the extent of lobular development in the virgin animal is strain dependent (12). Full alveolar development and maturation of the mammary epithelium take place during pregnancy and are dependent upon high circulating concentrations of progesterone, prolactin, and/or placental lactogen (12). Upon withdrawal of progesterone at parturition, lactation commences. Ongoing milk secretion continues during lactation under the influence of prolactin and oxytocin and ceases at weaning. When regular extraction of milk ceases, the alveolar epithelium undergoes apoptosis and remodeling, and the gland reverts to a prepubertal stage (12).

Since the mammary gland undergoes its functional morphogenesis in the young adult to adult stages and is very susceptible to tumorigenesis (13), it would be desirable to be able to manipulate its genetic complement at different developmental stages and study the effects of these changes. For this reason, we investigated the utility of adenovirus transduction in vivo to alter gene expression by using injection through the nipple to gain access to the epithelium from the exterior of the animal. Earlier investigators have taken advantage of intraductal injection techniques, using injections in goats and mice to study the permeability of the mammary epithelium to Na^+, Cl^-, and radiolabeled sucrose (8, 16).

DEAE-dextran-mediated transfection has been used to obtain human growth hormone expression in the guinea pig mammary gland after intraductal injection (5). However, the proportion of transfected cells was quite low. On the other hand, adenovirus transduction has proven to be a suitable method for efficient transduction of primary mammary cells in vitro in combination with mammary gland reconstitution to yield highly efficient gene transfer (18). In vivo, Jeng and coworkers injected an adenovirus vector coding for β-galactosidase into the rat mammary gland through the mammary duct and obtained significant expression of the gene (6). Yang et al. (24) obtained expression of LacZ in the mouse mammary gland in vivo after intraductal injection of an adenovirus vector. Although these studies demonstrated the effectiveness of adenovirus vectors, the issues of an inflammatory response and the efficiency of transduction have not been fully addressed.

We are primarily interested in the transition from pregnancy to lactation and sought a noninvasive, noninflammatory delivery system for introducing foreign genes into the mammary epithelium without transduction of the surrounding stroma. Our laboratory has perfected a technique of intraductal microinjection into the mouse mammary gland (14) and has used this

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technique to analyze tight junction regulation (15) and tight junction permeability relative to progesterone withdrawal and the presence of glucocorticoids (16) in the late pregnant mouse. We hypothesized that a similar intraductal injection technique could be used to obtain direct and localized transduction of the mammary epithelium with adenovirus vectors with minimal inflammation and little stress to the animal. The present study demonstrates that intraductal injection of a green fluorescent protein (GFP)-encoding adenovirus vector at late pregnancy leads to successful transduction of the epithelial cells in the proximal portion of the gland that lasts through parturition and into at least 5 days of lactation without inflammation. Although we examined transduction at late pregnancy most carefully, we also present data obtained at other stages of mammary gland development.

MATERIALS AND METHODS

Animals. CD-1 mice aged 5 to 9 weeks were purchased from Charles River Breeding Laboratory (Wilmington, Mass.) and maintained in the U.S. Department of Agriculture-approved Center for Laboratory Animal Care of the University of Colorado Health Sciences Center Animal Care Facility. Nulliparous, early pregnant (3 days; P3), late pregnant (17 days; P17), and lactating (4 days postparturition; L4) mice were used in this study. Pregnancies were timed by observing a vaginal plug (day 1 of pregnancy) after overnight residence with a male. The due date was calculated as 19 days after observation of the vaginal plug. Mice were housed under a 12-h light–12-h dark cycle and maintained on either breeder's chow (Teklad S-2335, no. 7004 mouse breeder diet; Harlan Teklad, Madison, Wis.) or a standard diet for postbreeding females (Teklad 22/5, no. 8640 rodent diet; Harlan Teklad, Madison, Wis.) and tap water ad libitum. All mice were anesthetized by intraperitoneal injection with avertin (125 to 250 mg/kg) and sacrificed by cervical dislocation. All procedures were approved by the Internal Animal Care and Use Committee of the University of Colorado.

Adenovirus vectors. Adenovirus vectors were grown in 293 cells, which are transformed by and express high levels of the adenovirus type 5 E1A and E1B proteins (4). A replication-defective adenovirus type 5 vector encoding enhanced, humanized, red-shifted green fluorescent protein under the control of the human cytomegalovirus major immediate-early promoter (Ad5GFP) was described previously (19).

Virus growth. Viruses were grown in 293 cells in Dulbecco's modified Eagle's medium containing high glucose and supplemented with 10% bovine calf serum. For growth of high-titer stocks, 293 cells were infected and harvested by centrifugation at the time of maximal cytopathic effect, and the virus was released by three cycles of freezing and thawing. Cell debris was pelleted, the supernatant was saved, and the pellet was resuspended in phosphate-buffered saline (PBS), frozen and thawed, and pelleted. The supernatant was combined with the first supernatant. The pellet was resuspended in PBS and pelleted. The supernatant was combined with the prior supernatants. The supernatants were overlaid on a step gradient consisting of 1.25 and 1.4 g of CsCl per ml in PBS and centrifuged for 50 min at 36,000 rpm in an SW41 rotor (Beckman). The virus band was collected by side puncture, diluted with 1.35 g of CsCl per ml in PBS, and centrifuged for 3 h at 65,000 rpm in a VTi65 rotor (Beckman). The virus band was collected by side puncture, dialyzed for 1 h each against three changes of adenovirus storage buffer (10 mM Tris-HCl [pH 8.0], 135 mM NaCl, 1 mM $MgCl_2$, 50% [vol/vol] glycerol), and stored at −20°C until use. The concentration of virus particles was determined from the absorption at 260 nm, with 1 A_{260} unit being equivalent to 10^{12} particles. Virus stocks were plaque titered on 293 cells.

Adenovirus microinjection. Ad5GFP microinjection was performed under avertin anesthesia at various stages of mammary gland development (Table 1). A stock of 2.7×10^8 PFU/ml was made in adenovirus storage buffer. Final doses (2.7×10^7 PFU for fourth mammary glands; 2×10^6 PFU for third mammary glands) were made by diluting the 2.7×10^8 PFU/ml stock with sterile filtered Ringer's solution (138 mM NaCl, 8.1 mM Na_2HPO_4, 1.2 mM K_2HPO_4, 2.7 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$). This dilution was made immediately before the microinjection to ensure the stability of the adenovirus. The solution was loaded into a 25-μl Wiretrol II disposable glass micropipette with a stainless steel plunger (no. 5-000-2050; Drummond Scientific Company, Broomall, Pa.). The end was drawn and fire-polished into a fine tip of 60 to 75 μm. By using a micromanipulator, the tip was gently inserted into the teat canal, and the solution was slowly ejected into the lumen of the either the third or fourth mammary gland as previously described (14). To evaluate the reliability of the injection technique, sterile filtered Ringer's solution was injected into contralateral control glands in some experiments.

TABLE 1. Experimental design

Reproductive stage		Total microinjection vol (μl)
Injection	End point[a]	
Nulliparous	3	5
P3	P6	80
P17	L2	80
P17	L5	80
P17	L10	80
P17	L11	80
P17	L15	80
P17	L27	80
L4	L7	80

[a] Days after injection, pregnancy day, or lactation day.

Determination of mammary epithelium permeability. Since increased [^{14}C]sucrose permeability is one of the hallmarks of mastitis (9), we used this isotope to determine mammary epithelium permeability. On the day of sacrifice, 2 μCi of [^{14}C]sucrose (Amersham, Buckinghamshire, United Kingdom) were lyophilized and dissolved in sterile Ringer's solution. Then 5 μl (nulliparous glands), 40 μl (fourth mammary glands), or 20 μl (third mammary glands) of this solution was injected intraductally into the lumens of adenovirus-injected and contralateral control mammary glands of each mouse under avertin anesthesia. The 10-μl blood samples were taken from the tail vein 5 min after each injection, and the amount of ^{14}C present was determined by liquid scintillation counting.

Preparation of tissue for freezing and histology. Injected experimental and contralateral control glands were excised and cut horizontally in half. One half of the gland was fixed in formalin, embedded in paraffin, and cut and stained with hematoxylin and eosin for histological purposes. The other half was cut into four to six smaller pieces and placed in aluminum foil molds filled with embedding medium (Tissue-Tek O.C.T. compound no. 4583; Sakura Finetek U.S.A., Inc, Torrance, Calif.) for frozen tissue specimens. The molds were flash frozen by immersion in an isopentane bath brought to its cooling point with liquid nitrogen.

Frozen sectioning and immunohistochemistry. Coverslips (Fisher Scientific no. 12-544-10) were treated with BD Cell-Tak and tissue adhesive (BD Biosciences no. 354240), rinsed, and stored overnight at 4°C. Then 12-μm sections were cut from the frozen molds with a cryostat at −32°C and collected onto the treated coverslips. The samples were placed at 37°C for 1 h and fixed in 2% paraformaldehyde (no. 00380; Polysciences, Inc., Warrington, Pa.) for 10 min.

After rinsing two to three times with PBS, the samples were treated with a blocking solution of 5% normal goat serum (#005-000-121; Jackson Immunoresearch, West Grove, Pa.) and 100 μg of saponin per ml (no. S4521; Sigma, St. Louis, Mo.). Samples were rinsed twice with PBS and incubated with the appropriate primary antibody for 1 h. A polyclonal antibody (7781) was made by using casein precipitated at pH 6.3 from mouse milk. Western blots showed specificity for mouse β-casein. Antibody against xanthine oxidase was generated against purified mouse xanthine oxidase in rabbit and purified on protein A-Sepharose (10). For nonantibody staining, samples were treated with wheat germ agglutinin conjugated to rhodamine (Molecular Probes; Eugene, Oreg.) to outline the luminal surface of mammary epithelial cells, and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma D-9542) diluted in PBS was used to stain for nuclei.

Samples incubated with a primary antibody were rinsed five times for 5 min each with PBS and treated with both donkey anti-rabbit IgG conjugated to rhodamine (Molecular Probes; Eugene, Oreg.) and DAPI diluted in PBS. Both antibody-treated and non-antibody-treated samples were then rinsed six times for 5 min each in PBS. Then 60 μl of mounting medium (ProLong antifade kit, no. P-7481; Molecular Probes, Eugene, Oreg.) were placed on slides (Fisherbrand Superfrost, no. 12-550-15; Fisher Scientific, Pittsburgh, Pa.), and the coverslips were carefully lowered onto each slide. The slides were kept in the dark overnight and then placed at 4°C for storage.

Determination of mastitis. We developed a mastitis scoring system to examine the inflammatory response in Ad5GFP-transduced mammary epithelium. Three randomly chosen fields from hematoxylin and eosin-stained slides were assessed by bright-field microscopy at 40× magnification from various samples for the number of polymorphonuclear cells, mononuclear cell infiltration (scored 0, 1, and 2), and epithelial organization, again on a subjective scale (scored 0, 1, and 2), where 1 represents some mononuclear cell infiltration and epithelial disor-

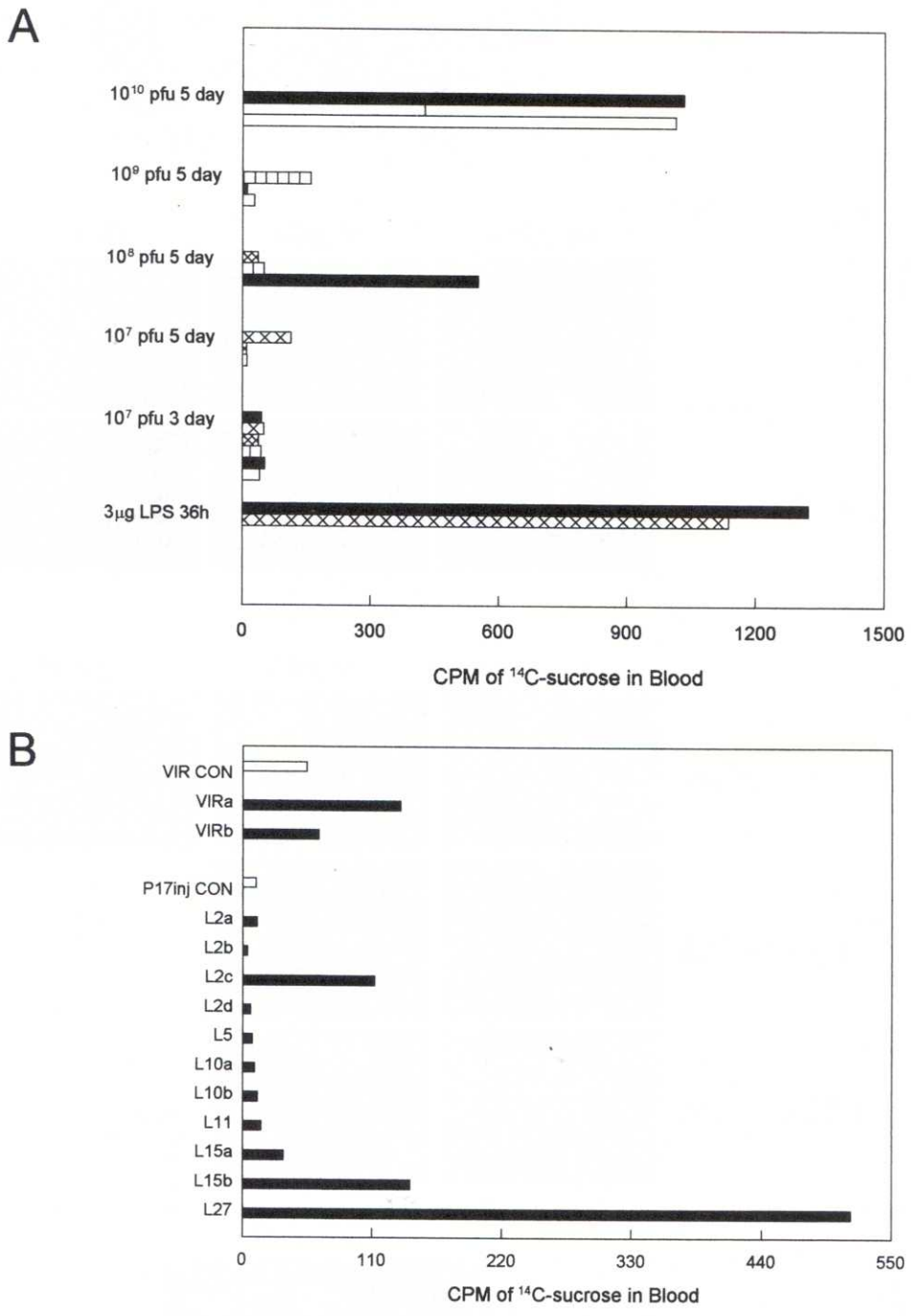


FIG. 1. Epithelial permeability as a function of Ad5GFP dose. (A) Mice were injected at P17 with the stated doses of Ad5GFP, and the permeability of the mammary epithelium was determined by injecting [^{14}C]sucrose intraductally 3 or 5 days postpartum and measuring the amount of ^{14}C in 10 μl of blood. Lipopolysaccharide was injected as a positive control for increased permeability. Each bar represents a different mouse. (B) [^{14}C]sucrose permeability in mice injected at various reproductive stages. [^{14}C]sucrose was injected intraductally into the third mammary glands of nulliparous mice (VIRa and VIRb) and P17 mice at various stages after Ad5GFP injection (see Table 1). Each bar represents an individual mouse. Nulliparous control glands (VIR CON; $n = 5$) received vehicle only. The amounts of [^{14}C]sucrose measured in the blood after injection of P17 control glands (noninjected; $n = 11$) contralateral to Ad5GFP-injected glands (P17inj CON) were averaged.

ganization and 2 represents significant mononuclear cell infiltration and epithelial disorganization. To obtain a mastitis index that provides equal weighting of all three measurements, we used the equation MI = P/4 + MC + EO, where MI is the mastitis index, P is the average number of polymorphonuclear cells, MC is the mononuclear cell infiltrate, and EO is the average epithelial organization score. Because 9 was the maximal polymorphonuclear cell count per field, we chose the value 4 to bring this score in line with the others. Thus, the mastitis score varied between 0 and 6. Three independent observers, one of whom was blinded to the treatment, evaluated each slide with similar results.

Microscopy and quantification of transduction. For an initial assessment of Ad5GFP transduction, the glands were visualized under a Nikon dissecting microscope under fluorescent light. Digital images were captured by using Ax-

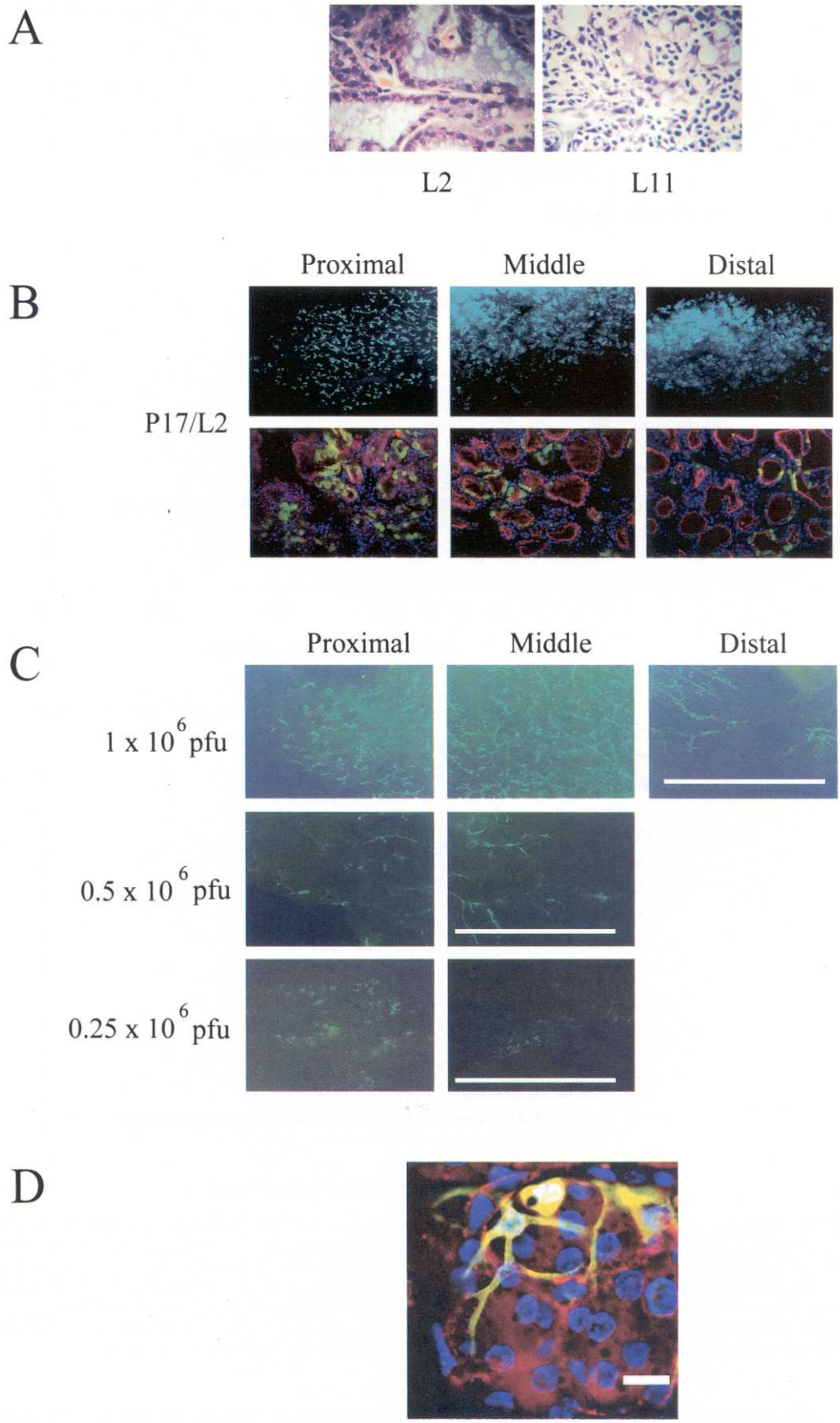


FIG. 2. Histological evidence of mastitis and extent of Ad5GFP transduction in nulliparous animals and animals injected during late pregnancy. (A) Hematoxylin and eosin-stained sections of third mammary glands from mice injected at day 17 of pregnancy (P17) and sacrificed on days 2 (L2) and 11 (L11) of lactation. (B) Initial assessment of transduction in whole glands in regions relative to the teat (proximal, middle, and distal) examined under fluorescent light (upper panel), and 12-μm sections examined under a confocal microscope (lower panel). (C) Whole glands from three individual nulliparous mice injected with different doses of Ad5GFP examined under fluorescent light. Bars, 5 mm. (D) Transduced myoepithelial cell. Bar, 20 μm.

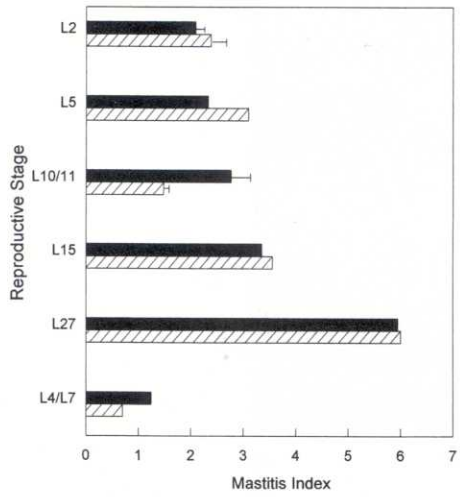


FIG. 3. Evaluation of mastitis. Data represent animals injected with Ad5GFP at P17 and sacrificed at various stages of mammary gland development (solid bars) and their respective controls (noninjected contralateral glands; hatched bars): L2 (n = 3), L5 (n = 2), L10/11 (n = 3), L15 (n = 2), L27 (n = 2), and L4/L7 (n = 2). Glands were assessed at 40× magnification by bright-field microscopy as described in Materials and Methods. See Materials and Methods for definition of the mastitis index.

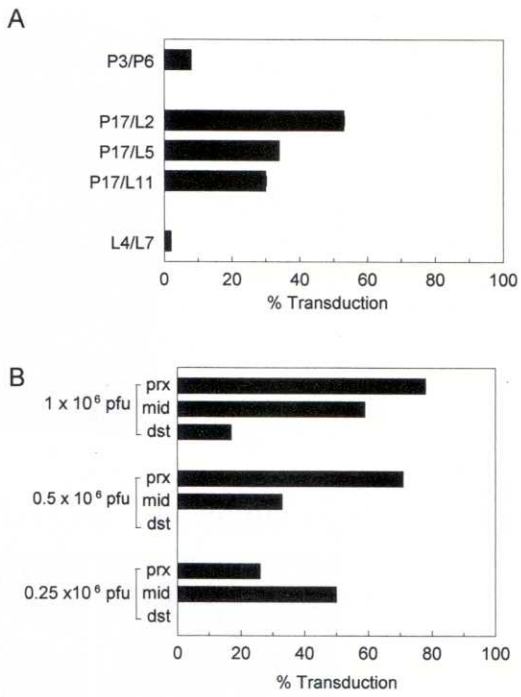


FIG. 4. Percent transduction in early pregnant, late pregnant, and nulliparous glands injected with AdGFP. (A) Percentage of epithelial structures (ducts and alveoli) showing transduction during mammary gland development. Each bar represents an average for three regions of the mammary gland (proximal, medial, and distal relative to the teat) at various stages of development: P3/P6 (n = 2), L2 (n = 3), L5 (n = 2), L10/11 (n = 3), and L4/L7 (n = 2). (B) Percent transduction of epithelial structures in nulliparous mice at different doses of Ad5GFP. Percentage of transduced epithelial structures was determined manually by counting the number of transduced and nontransduced epithelial structures in nulliparous mice injected with different doses of Ad5GFP: 1×10^6 PFU (n = 2), 0.5×10^6 PFU (n = 2), and 0.25×10^6 PFU (n = 1). Each bar represents the average for three sections in each segment (proximal [prx], medial [mid], and distal [dst] to the teat) of the gland.

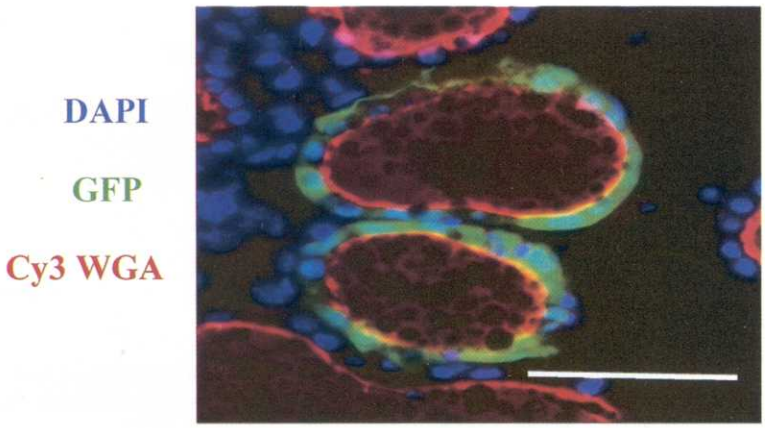
ioVision software (Carl Zeiss, Inc., North America). A Nikon fluorescent confocal microscope was used to visualize immunohistochemical samples, and Slidebook software (Intelligent Imaging Innovations, Denver, Colo.) was used to capture the higher-power images. Percent transduction of mammary alveoli and/or epithelial structures was determined by manually counting transduced and nontransduced structures in three segments of the gland relative to the teat (proximal, medial, and distal). To determine the extent of transduction of epithelial cells, images were captured at identical magnifications and exposure times and quantified by using a masking program in Slidebook that was capable of determining either the proportion of transduced epithelium or the proportion of transduced nuclei by using DAPI-stained nuclei as a reference.

RESULTS

Adenovirus dosage determination. To determine the maximal dose of adenovirus vector that could be injected without induction of inflammation, various amounts of Ad5GFP were injected intraductally into the fourth mammary glands of P17 mice. We assessed permeability by injecting [^{14}C]sucrose intraductally and measuring its level in the blood (15, 16). Because lipopolysaccharide induces massive mastitis (20), we injected it as a positive control. As the data in Fig. 1A show, high blood levels of sucrose were associated with injection of lipopolysaccharide. An adenovirus dose of 10^{10} PFU also led to high sucrose permeability (Fig. 1A) as well as histological signs of mastitis (data not shown). Sucrose permeability was relatively low with adenovirus doses of 10^8 and 10^9 PFU (Fig. 1A), with the exception of one sample. At a dose of 10^7 PFU, epithelial permeability remained near baseline (Fig. 1A) and the tissue showed no signs of mastitis (data not shown). A dose between 10^7 and 10^8 PFU (2.7×10^7 PFU) was determined to be safe for the fourth mammary gland of the mouse. For experiments in which we used the third gland, which is about one-fifth the size of the fourth gland, we injected 2×10^6 PFU.

Duration of transduction. In the next set of experiments, we used the third mammary gland to assess the amount of damage incurred by the mammary epithelium at various times after injection in a late pregnant mouse. In some cases, we evaluated the proximal, medial, and distal portions of the gland relative to the teat. As before, intraductal [^{14}C]sucrose injection was used to measure the permeability of the mammary epithelium (Fig. 1B), and mastitis was evaluated in hematoxylin and eosin-stained sections (Fig. 2A). Figure 2A shows an L2 mammary gland with no detectable mastitis. An island of massive mononuclear cell infiltration observed in a gland of a mouse sacrificed on lactation day 11 (L11), 12 days after adenovirus treatment, is also shown in the figure. A mastitis scoring system was designed to provide a semiquantitative measure of the extent of damage. This score, the mastitis index (measured as described in Materials and Methods), is shown for various times after injection in Fig. 3 and, in general, was not different from the index in the contralateral gland The L10 results were confirmed by three observers, but the [^{14}C]sucrose permeability data suggest that the degree of mastitis in the Ad5GFP-injected gland was small. Both the sucrose permeability data and the mastitis score show that an optimal dose of adenovirus produces little damage when injected into late pregnant mice

A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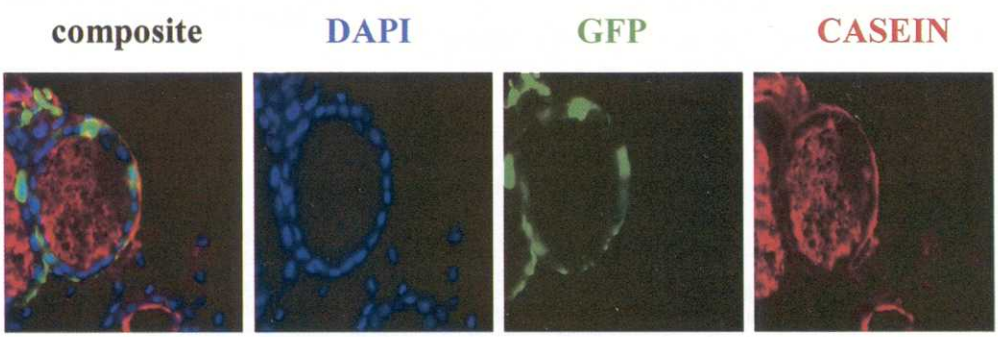
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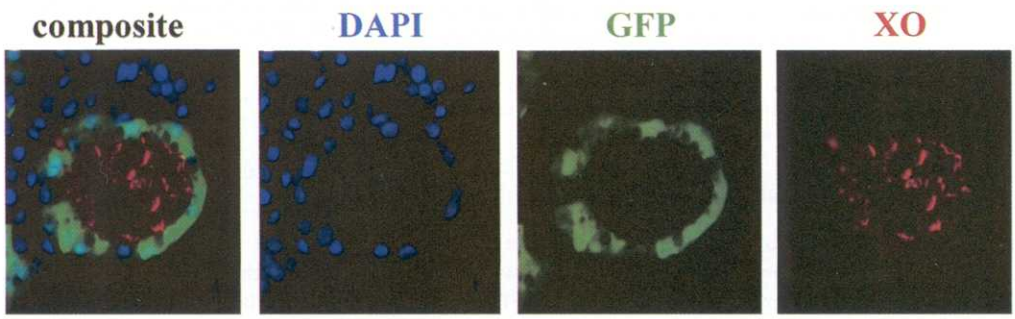
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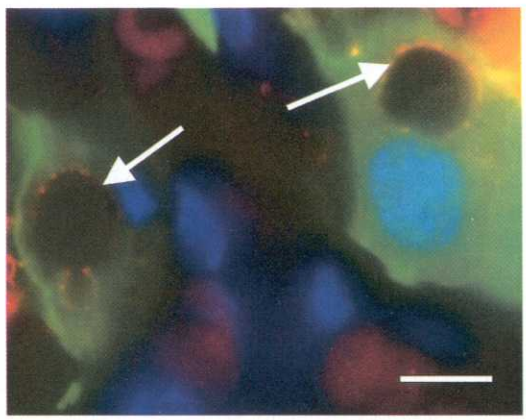

and left up to 5 days or longer into lactation (Fig. 1B and 3). By L27, the gland becomes disorganized due to involutional remodeling (17).

We also examined Ad5GFP transduction in mammary glands from nulliparous mice. We found that volumes larger than 5 μl burst the small ductal system in these glands (data not shown), so all adenovirus injections were restricted to this volume. As a control, 5 μl of sterile-filtered Ringer's solution was injected into the contralateral gland. Three independent observers, one blinded, concluded that there were no gross differences between the control and injected glands and that mastitis appeared relatively minimal (data not shown). The extent of transduction in early pregnant and lactating glands was very low (see below), so mastitis was not formally evaluated in these conditions.

Extent of adenovirus transduction. We viewed transduced mammary glands at low magnification to obtain an initial assessment of the amount of Ad5GFP transduction (Fig. 2B, upper panel, and 2C). Although transduction appeared to be concentrated in areas proximal to the teat or in the middle of the gland in most nulliparous and pregnant animals, some glands did exhibit widespread transduction, as shown in Fig. 2B and 2C (upper panels). We assessed mammary sections from pregnant and lactating mice by using a 20× confocal view of 12-μm-thick frozen sections, first counting the proportion of ducts and alveoli that showed some transduction (Fig. 2B, lower panel). This analysis showed that many but not all alveolar structures were transduced. Less than 10% of the ductal and alveolar structures of glands from early pregnant mice showed some transduction, while approximately 30 to 50% of the structures were transduced when the virus was injected in glands from late pregnant mice (Fig. 4A). Very low transduction was achieved in glands from Ad5GFP-injected lactating mice (Fig. 4A).

To further investigate the extent of alveolar transduction, we used the masking function of Slidebook (see Materials and Methods). By using glands injected at P17 and sacrificed on day 2 of lactation, we were able to delineate specific cellular areas of transduction and calculate the proportion of total epithelium transduced in each alveolar structure. With this approach, we found that the percentage of transduced epithelium was highly variable between two different mice (data not shown). For example, approximately 25% of the total epithelium was transduced at this stage of mammary gland development in areas proximal to the teat in one animal, and only 7% of the total epithelium was transduced in the same area in another mouse. Areas medial and distal to the teat had lower percentages of total transduced epithelium that also varied greatly between animals (data not shown).

To get an approximate measure of the efficiency of transduction, an estimation of the number of epithelial cells in the third mammary gland was made and compared with the frequency of transduction. By using the DNA content of the gland as a starting point, it was estimated that there were approximately 3×10^7 epithelial cells. Approximately 20%, or 6×10^6, of these cells were transduced in the most highly transduced gland when 2×10^6 PFU of Ad5GFP were injected. The ratio of transducing units to PFU was near 5:1, so approximately 10^7 transducing units were injected. This analysis suggests that greater than half of the injected vector transduced epithelial cells, supporting the idea that the process is relatively efficient during the late stage of pregnancy in the mammary gland.

The mammary gland consists of an inner layer of ductal cells and alveoli and an outer contractile monolayer of myoepithelial cells closely attached to the basement membrane. The myoepithelial cells extend laterally along ducts and form a basket-like sheath around both ducts and alveoli (13). In response to oxytocin binding to specific receptors, myoepithelial cells contract and expel milk from the alveoli into the ducts and eventually out of the gland. The presence of GFP in myoepithelial cells in glands transduced in late pregnancy (Fig. 2D) suggests that viral particles were able to traverse the paracellular compartments of the mammary gland or that some myoepithelial cells were exposed at the luminal surface. The presence of GFP in myoepithelial cells also raises the possibility that the basolateral surface of the mammary epithelium is accessible during late pregnancy, when the paracellular spaces are open to large molecules, allowing adenovirus access to its receptor (1, 23, 27).

Epithelial structures of nulliparous mice can also be transduced by Ad5GFP (Fig. 2C and 4B). Portions of the gland proximal to the teat were highly transduced after injection with relatively high doses of virus, but the extent of transduction was greatly reduced with decreasing doses (Fig. 2C and 4B). It is possible that adenovirus transduction can be used experimentally in nulliparous animals with small volumes and careful attention to optimizing the dose.

Functional competence of transduced cells. The data presented to this point show that mammary epithelial cells can be transduced with Ad5GFP during pregnancy and can be maintained well into lactation without inflammation. However, in order to utilize adenovirus microinjection as an effective method of changing gene expression, transduced cells must retain functional integrity. Two distinct pathways of cellular milk synthesis, milk protein secretion and milk fat secretion, can be assessed morphologically. Transduced alveoli displayed normal morphology, produced and secreted milk fat globules, and were laden with milk, which was stained red in the lumen of the alveoli in Fig. 5A. Casein, a milk protein, was also produced and was detected in the lumen of transduced alveoli (Fig. 5B). Xanthine oxidase has been shown to redistribute from the cytoplasm to the surface of emerging milk fat globules during pregnancy at the onset of lactation, and this redistribution is thought to be essential for milk fat globule release (J.

FIG. 5. Maintenance of function in mammary glands transduced with Ad5GFP. (A) Two nearly completely transduced (green) alveoli showing milk oligosaccharides stained with rhodamine-wheat germ agglutinin (red), nuclei stained with DAPI (blue), and surrounding milk lipid droplets. These alveoli appear morphologically normal. Bar, 100 μm. (B) A 20× view of lumens of transduced (green) alveoli stained with anticasein antibody (red); the nuclei were stained with DAPI (blue). (C) A 20× and (D) a 100× view of fat droplets in transduced (green) alveoli rimmed with xanthine oxidase (XO) as shown by stain (red) with an anti-xanthine oxidase antibody; the nuclei are stained with DAPI (blue). Arrows indicate milk fat droplets rimmed with xanthine oxidase in panel D. Bar, 20 μm.

McManaman, personal communication). This localization of xanthine oxidase was maintained in transduced cells (Fig. 5C and 5D). Thus, the transduced cells appeared to be fully functional.

DISCUSSION

Adenovirus vectors have been used to transduce a variety of organs in a number of animal species. Mice have been especially popular as models to study the effects of adenovirus vectors. Intraductal injection of adenovirus vectors provides a versatile method of altering gene expression in both the luminal and, to some extent, the myoepithelial cells of mouse mammary glands. In no case did we find transduction in the interstitial spaces of the mammary glands that we studied. It is possible to transduce nulliparous glands (Fig. 2C and 4B), even though these glands are immature compared to late pregnant glands and are particularly smaller in size. Although adenovirus can be used to transduce the epithelium at any stage of mammary gland development except lactation, the technique has proven to be most effective during the switch from pregnancy to lactation (Fig. 2B, 3, 4A, and 5A). Little mastitis was observed up to a week to 10 days after transduction with an optimal dose of virus (Fig. 1 and 3). The extent of transduction was highly variable within regions of the gland relative to the teat and may not generate enough material for biochemical analysis of transduced cells. Nevertheless, morphological studies may still be possible with neighboring nontransduced cells as controls.

We had less success with adenovirus transduction at other stages of mammary gland development. Transduction during early pregnancy was relatively low (Fig. 4A), possibly because the epithelium is turning over rapidly at this developmental stage (2). Transduction during lactation (Fig. 4A) was rarely successful. Our laboratory has previously shown that tight junction permeability is very low during lactation (16), preventing solute passage through the paracellular pathway to the basal surface of the gland, where the adenovirus receptor may be localized (23, 27) There is also evidence that the coxsackie and adenovirus receptor is a component of the tight junction complex and that its localization within this complex impedes viral transduction (3).

Taking these factors into consideration, it is possible that the change in tight junction permeability that accompanies secretory activation is responsible for the low efficacy of adenovirus transduction during lactation. Also, during lactation, the high concentration of milk proteins may adsorb the viral particles. The density of adenovirus receptors may also differ at different stages of mammary gland development with changes in endocrine state, and these differences may influence the efficacy of adenovirus transduction (7). Thorough investigations with such techniques as in situ hybridization and adenovirus receptor density studies in mouse mammary glands at various stages of development are needed to properly address these issues. Nevertheless, adenovirus vectors appear to be effective in mediating the transduction of functional genes into mouse mammary epithelial cells, particularly ain late pregnancy, when the tight junctions between epithelial cells are open to the passage of large molecules (15).

Our results also demonstrate that transduction with Ad5GFP does not disrupt normal mammary epithelial cell morphology and function (Fig. 5). Adenovirus transduction did not disrupt either of two major and distinct synthetic pathways for cellular milk secretion, milk protein secretion and milk fat globule formation. These data clearly show that adenovirus transduction can be used to alter gene expression and to study luminal cell function in the mouse mammary epithelium.

Adenovirus vectors used in sufficient amounts to efficiently transduce target tissues generally induce a strong inflammatory response that typically reaches a high level within 4 to 7 days of vector introduction in a variety of immunocompetent mouse strains. Inflammation occurs within targeted organs transduced via the bloodstream, such as the liver (25), as well as when the mucosal surface of an organ is targeted, such as in the lung (26). The inflammatory response both limits the duration of expression and leads to an immune response that limits successful reuse of the vector. The relatively rapid induction of inflammation has limited the usefulness of adenovirus vectors for studies of altered gene expression in most tissues. Thus, the findings presented here are somewhat surprising.

It is not clear why the induction of inflammation is delayed when adenovirus vectors are used in sufficient amounts to transduce a significant fraction of the mammary epithelium. It is possible that this delay is due to the use of outbred CD-1 mice. Adult CD-1 mice have been successfully transduced for extended periods of time with adenovirus vectors (22), and neonatal CD-1 mice did not exhibit inflammation after injection with an adenovirus vector (21), raising the possibility that CD-1 mice may exhibit reduced inflammatory and immune responses to adenovirus vectors relative to other mouse strains. Alternatively, it may be that pregnancy reduced the immunocompetence and inflammatory response of the mice. Regardless, the delayed onset of inflammation means that adenovirus vectors can be effectively used as a tool for altering gene expression in the mammary gland of CD-1 mice.

The usefulness of first-generation adenovirus vectors in transduction of the mammary epithelium of late pregnancy means that it is not necessary to undertake the substantial efforts required for the use of helper-dependent, or "gutless," vectors. However, it remains a possibility that the use of second-generation vectors made replication incompetent through deletion of a viral gene essential for viral DNA replication, such as the terminal protein gene (11), will lead to a reduction in the low level of inflammation or extend the time prior to the appearance of inflammation, extending their usefulness. Since such vectors are relatively easy to construct and grow, it may be worthwhile to test their effectiveness in transducing the mammary epithelium.

Intraductal microinjection of adenovirus vectors should aid in studies of a variety of genes of interest in the mammary epithelium. Since adenovirus transduction appears to be confined to the mammary epithelium, these methods provide a technique to target genes of interest to this tissue compartment. Potentially, these techniques could be used for drug, hormone, or protein delivery to milk on a short-term basis. Currently, our laboratory is preparing to use the methods described here to examine the regulation of milk synthesis and secretion in the mouse mammary epithelium. Recombinant adenovirus constructs could potentially target genes responsible for certain types of breast cancers. The procedures de-

scribed here provide a means of studying the efficacy of such vectors.

ACKNOWLEDGMENTS

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TRANSDUCTION OF THE MAMMARY EPITHELIUM WITH ADENOVIRAL VECTORS

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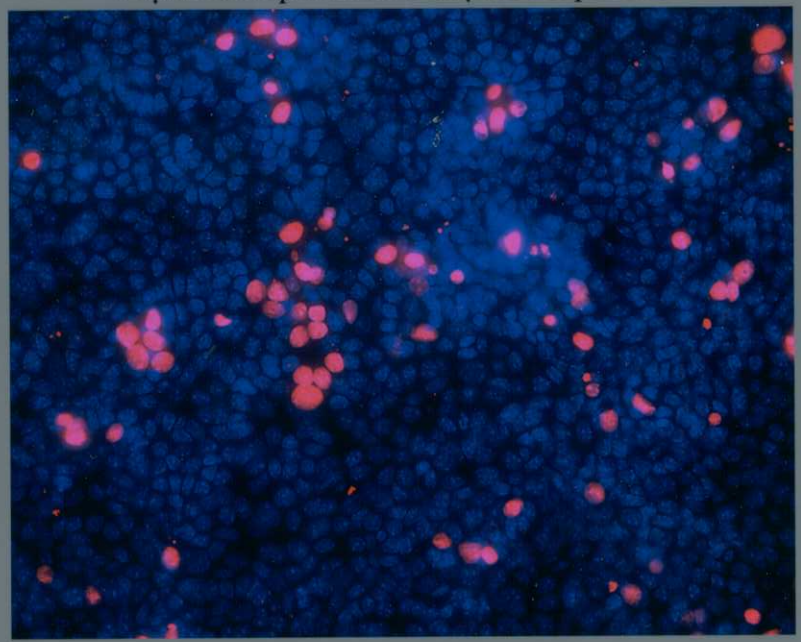
ABSTRACT:
Up the teat microinjection of adenoviral vectors presents a non-invasive, non-inflammatory delivery system to study gene expression in the mammary epithelium. An E1/E3 deleted adenoviral vector (human adenovirus type 5) encoded with either LacZ or GFP reporter genes was injected into the fourth (LacZ) or third (GFP) mammary gland of mice at various stages of mammary gland development. 14C-sucrose was injected intraductally on the day of sacrifice to test the status of tight junctions and glands were excised to examine evidence of mastitis. Doses of 107 pfu (fourth mammary gland) or 2.6 x 106 pfu (third mammary gland) injected into day 17 pregnant mice showed minimal inflammation after 3 days. However, significant mastitis resulted after 7-10 days even with optimal doses of the adenovirus. Up to 40% of alveoli can be transduced at day 17 of pregnancy and up to 25% of the total epithelium can be transduced in the portion of the gland proximal to the teat. Although adenovirus can transduce the epithelium at any stage of mammary gland development, transduction gives the least inflammation in the late pregnant animal and can be maintained into early lactation. These findings demonstrate that up the teat microinjection of adenoviral vectors provides a versatile method of changing gene expression in cells of the mammary epithelium.

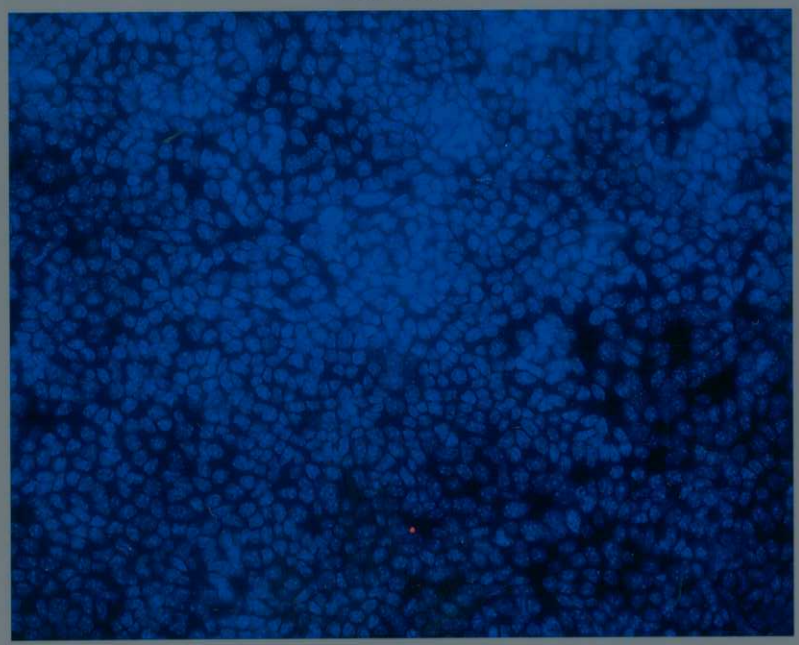
Figure 1

Etoposide and Camptothecin together resulted in programmed cell death by 69 hours.

30μM camptothecin 50μM etoposide



Control



The TUNEL staining shown here in red demonstrates dying nuclei. The healthy nuclei are stained blue

Figure 2

Fascin appears to cluster around the nuclei of topoisomerase inhibited cells

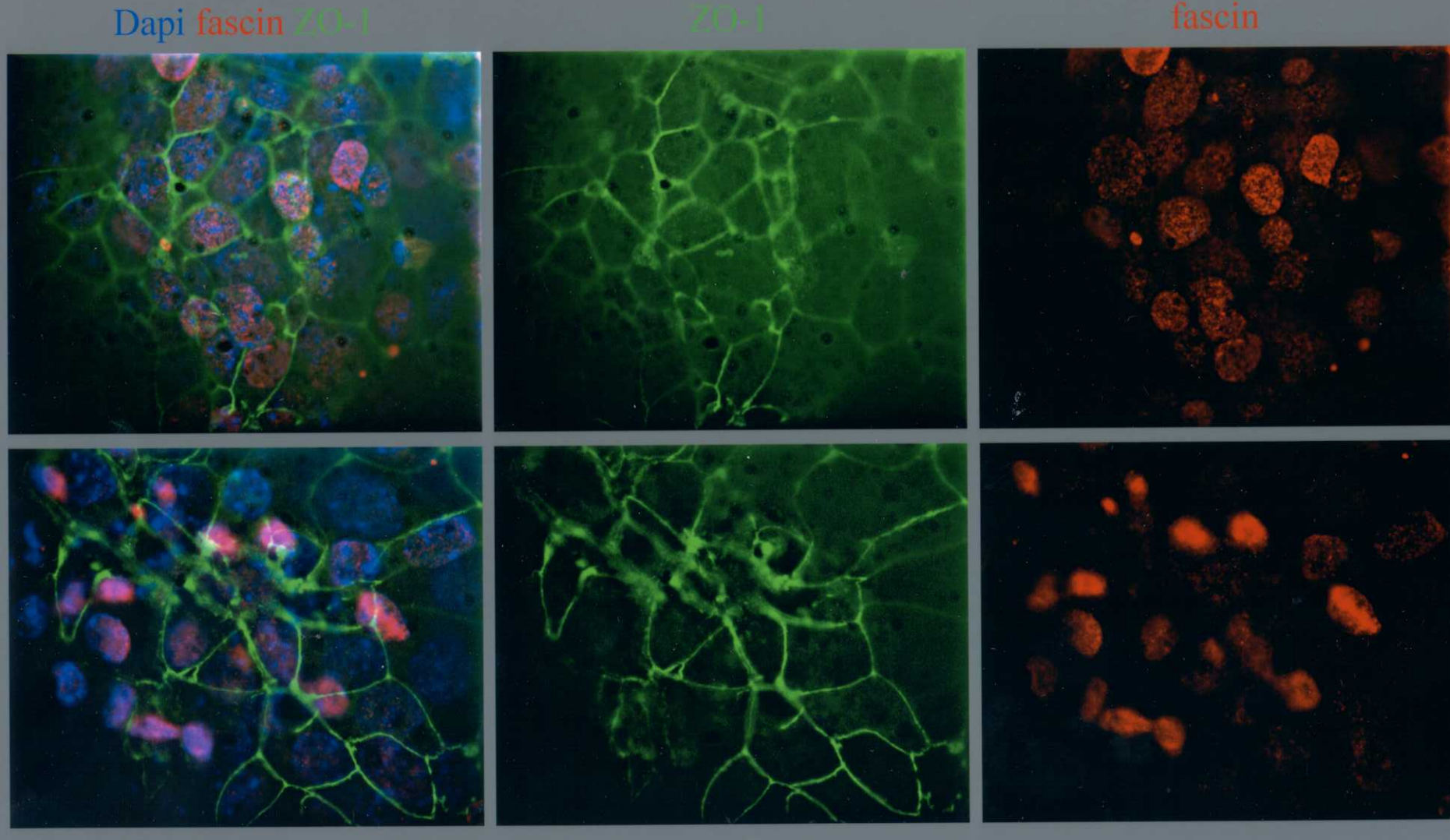


Figure 3

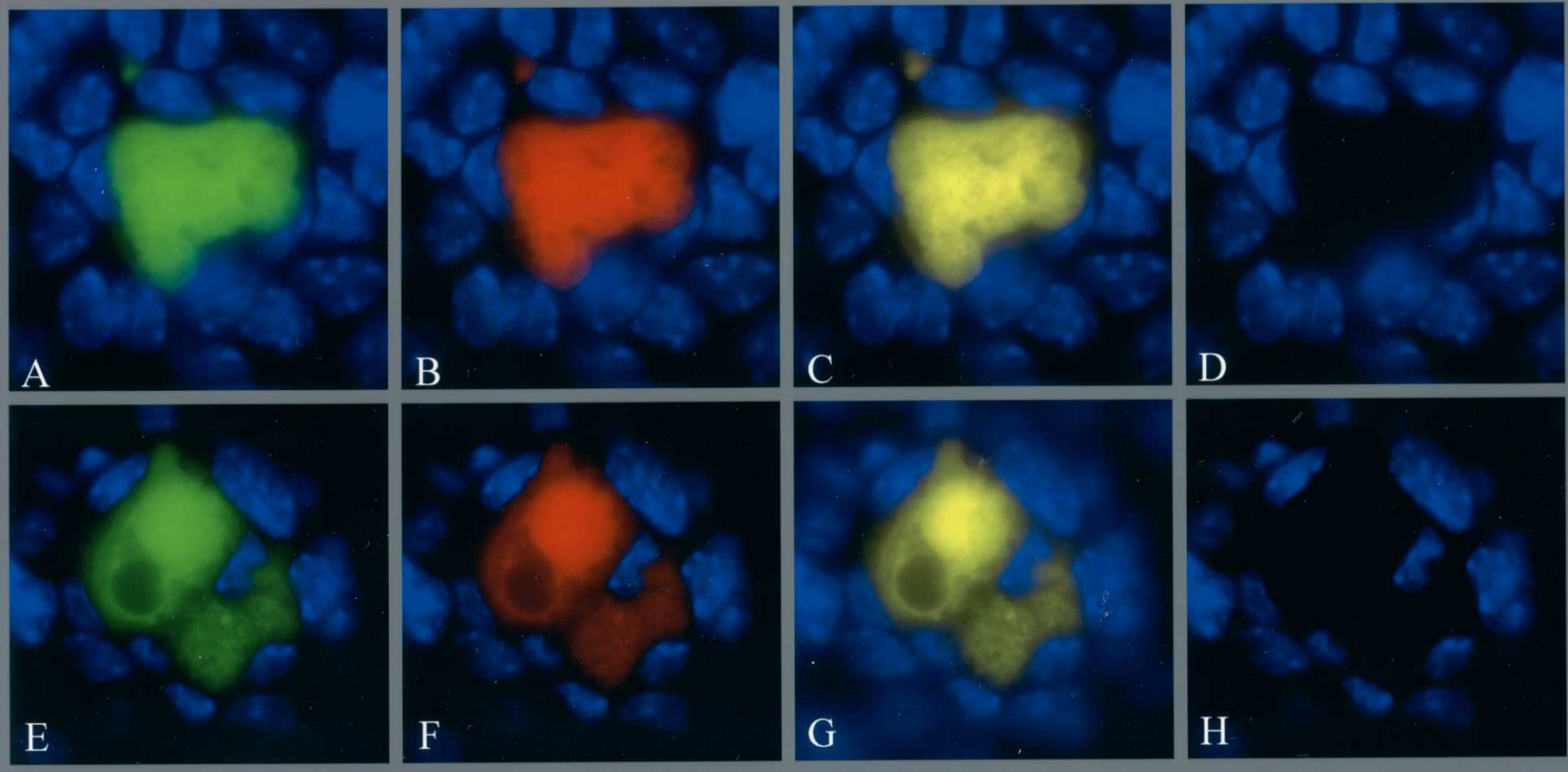


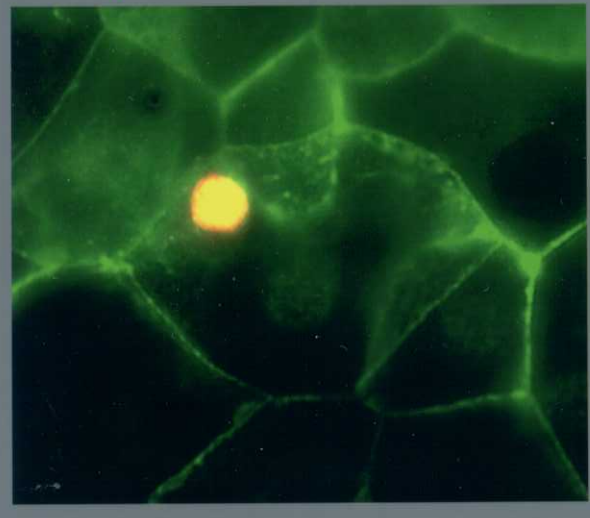
Figure3. Effect of the FLAG tagged N-terminally truncated occludin construct on the localization of fascin 48 hours post transduction:

Filter grown CIT3 cells were treated with a multiplicity of infection (moi) of ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding the occludin transgene and GFP. The filters were processed for immunofluorescence 48 hours following viral treatment. Filters were incubated with a mouse monoclonal antibody against fascin and then treated with a Cy3 (red) conjugated anti-mouse secondary antibody. Antibody treated filters were then stained with dapi nuclear dye (blue): A-H = 100x; A-D= same cells; E-H = same cells; A and E = GFP and dapi; B and F = fascin and dapi; C and G = fascin, GFP, and dapi; D and H = dapi.

Figure 4

β-catenin localizes to a perinuclear region in dying cells. This appears to be different than the truncated occludin results

β-cat TUNEL



β-catenin

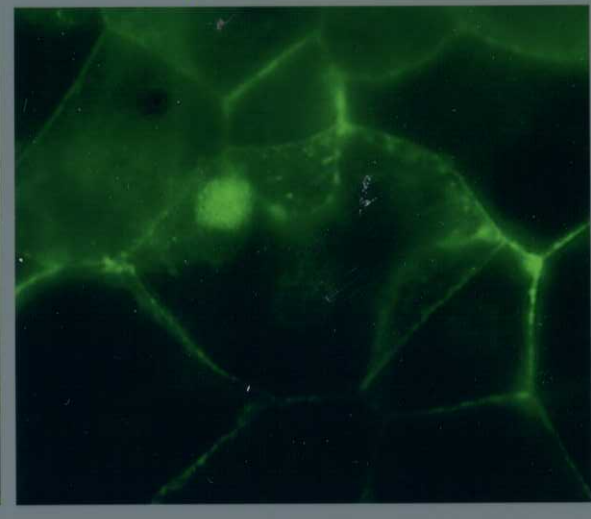


Figure 5

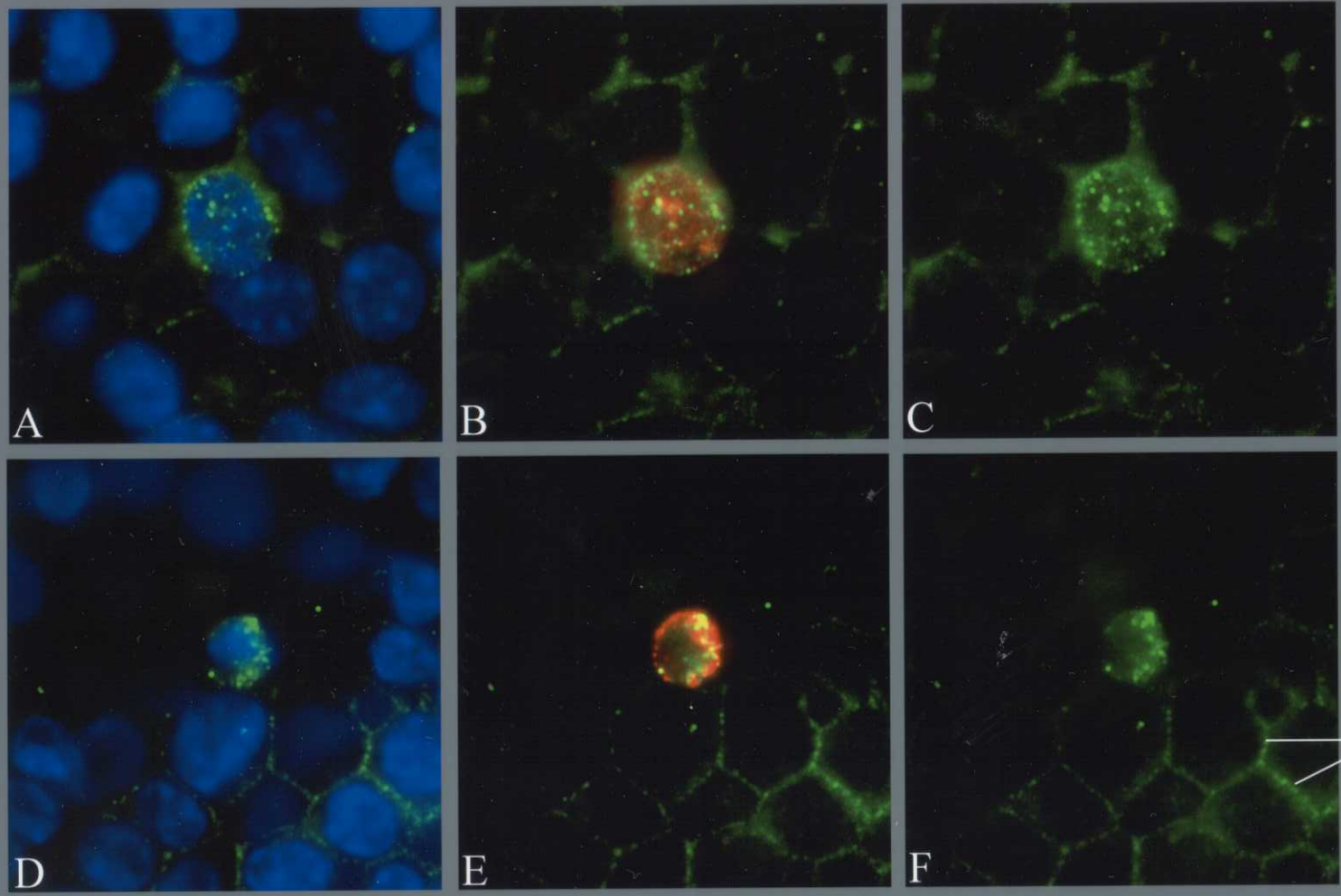
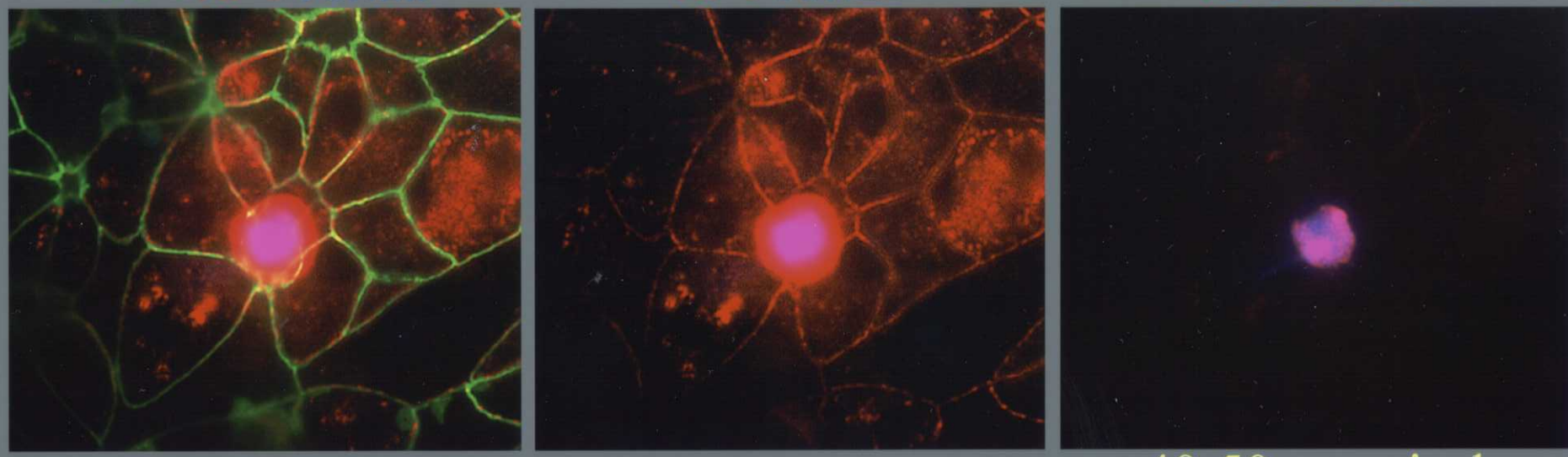


Figure 5. Effect of the FLAG tagged N-terminally truncated occludin construct on the localization of β-catenin:

Filter grown CIT3 cells were treated with a multiplicity of infection (moi) of ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding the occludin transgene and GFP. The filters were processed for immunofluorescence 48 hours following viral treatment. Filters were incubated with a mouse monoclonal antibody against FLAG and a goat polyclonal antibody againstβ-catenin. Sections were then treated with Cy3 (red) conjugated anti-mouse and Cy5 (shown in green) conjugated anti-goat secondary antibodies. Antibody treated filters were then stained with dapi nuclear dye (blue): A-F = 100x; A-C = same cell; D-F = same cell; A and D = β-catenin and dapi; B and E = FLAG and β -catenin; C and F = β -catenin.

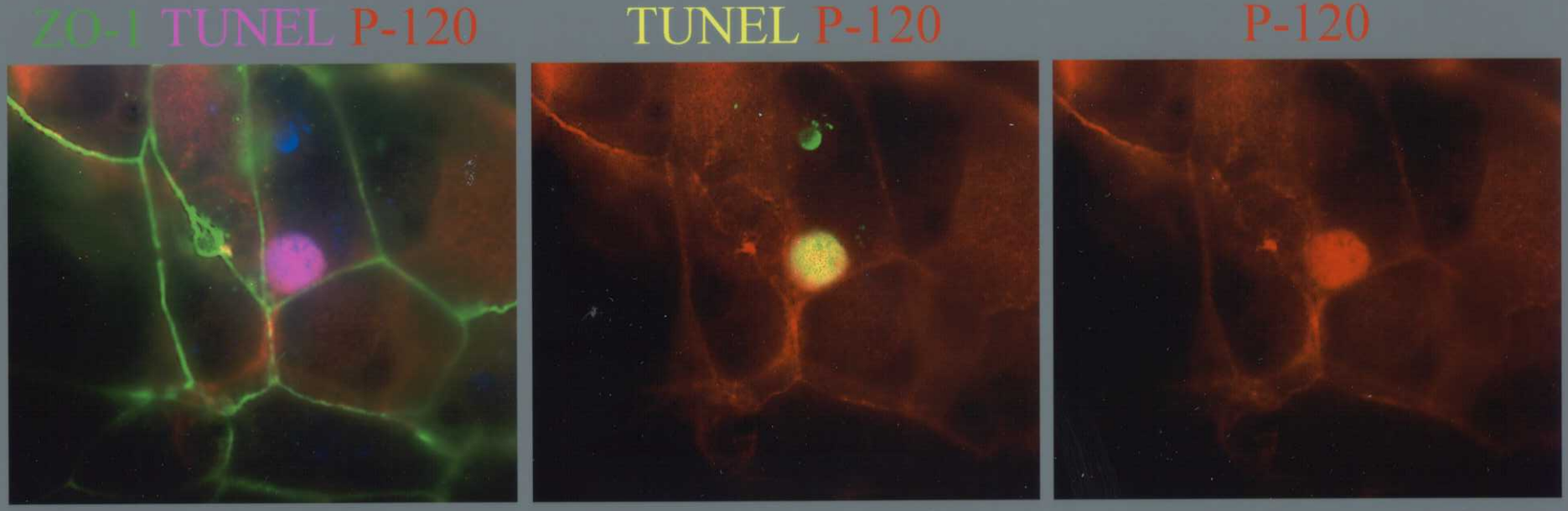
Figure 6

AF-6 associates with pycnotic nuclei during apoptotic ejection



40-50μm apical from left panels

P-120 catenin has a similar distribution to β-catenin during topoisomerase inhibition



Cleaved Caspase-3 staining 48hr after pup removal

nuclei actin activated caspase-3